EXHIBIT B

2)

1

Repeat w.t. peptide binding Assay.

5 x 10^5 T2 cells / well in RPMI + 5% Boiled FCS.
Add peptides to a final Conc. of 100 μM.
Leave to binding at 37°C o/N
Wash x 1. with PBS + 2% FCS.
Apply mAb HB54. HB117 30' on ice
Wash x 3
Apply GaM FITC (1/250) 30' on ice
Wash x 3
Resuspend cells in PBS : (400 μl).

FACS Analysis.

Results. Good.

peptide — 2nd Ab only	Mean: HB54 513	HB117
	37.70	42.32
HPV E7/86-93	109.20	118.12
HBV	146.86	220.67
WT-1 /10-	81.62	144.38 ++
WT-1 /17-	43.79	57.04 +
WT1 /126-	69.78	130.79 ++
WT1 /187-	93.01	136.27 ++
WT1 /225-	70.03	97.56 +
WT1 /235-	95.63	105.90 ++
WT1 /280-	45.66	76.42 +
WT 1/441-	137.52	172.31 ++

A2 Binding Titration.

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peptides WT1

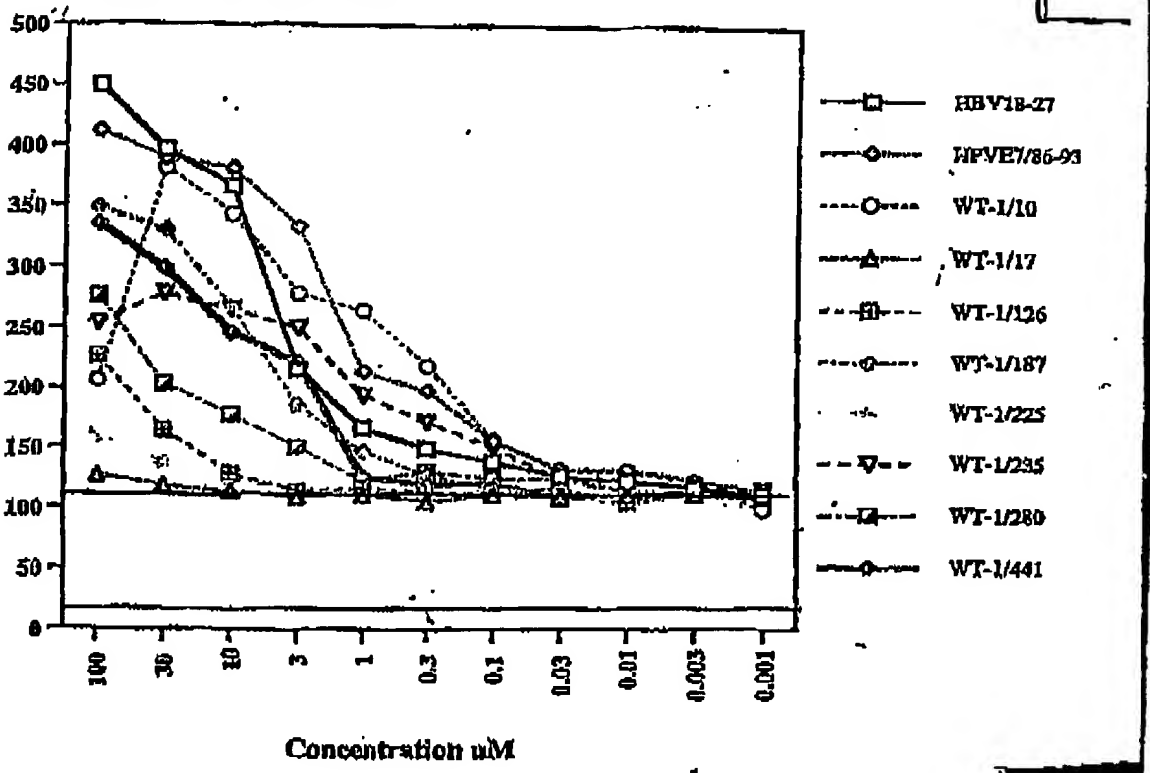
10.
17
126
187
225
235
280
441
HPVE7 E6-93
HBV 17-28

Dilute peptide in RPMI +5% Boiled FCS in 96 well plate (U bottomed). from 100μM of first column to column 11 0.0001 μM) in 50μl medium.
Add 50μl of T2 cells at a conc of 6×10^6/ml ie. 5×10^5/well mix well.
37°C overnight.
wash x1 in PBC (cold) + 1% FCS
stain in HB117 and HB54 (neat culture sup) 100μl for 30' on ice.
wash x 3
Apply Ga M Ig FITC at 1/250 (100μl).
for 30' on ice
wash x 3.
Analyse on FACS

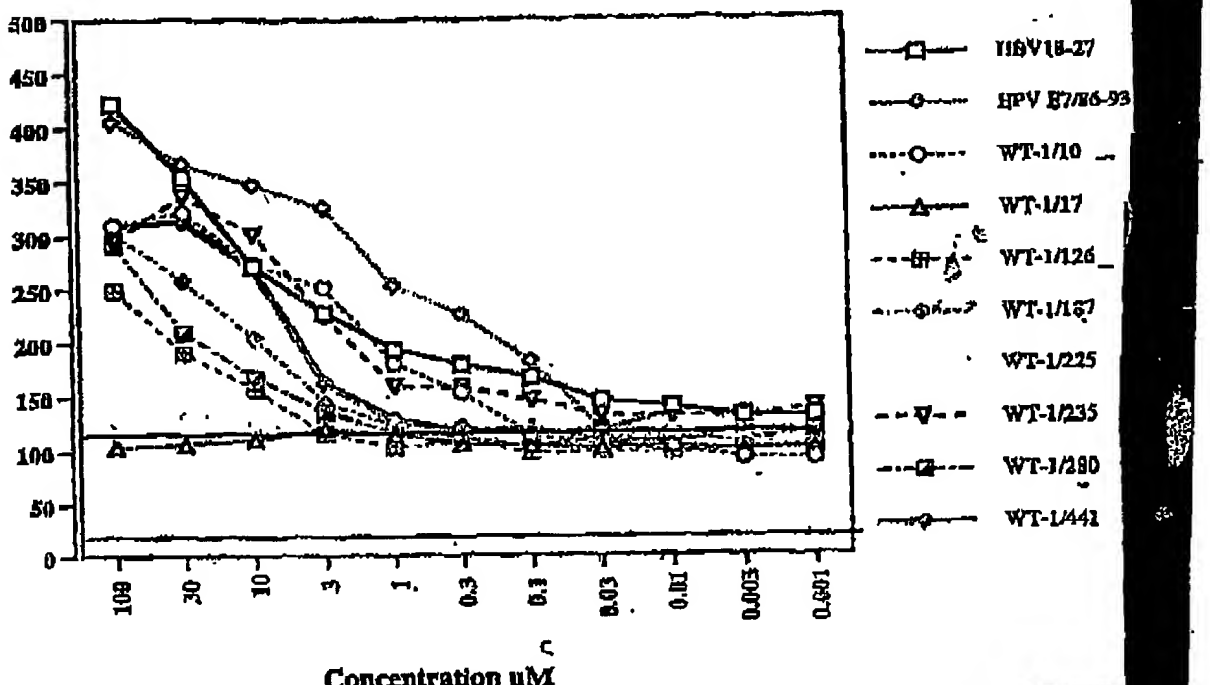
Results: Good see attached sheets.

3

WT-1 peptides binding to A2 molecules on T2 cells
----Stained with HB54 mAb



Binding of WT-1 peptides to A2 molecules on T2 cells
----Stained with HB117 mAb



3.)

4

Setting up Allo- & Self restricted peptide CTL lines

PBMC: LG3 A_2^- Allo

LG4 A_2^+ Self

peptide: WT1 10, 235, 441 and C228 (+ve Control).

Method: as Left — use DC from LG4 as stimulator

Restimulate with peptide loaded CIRA2 cells. (50 μl).

Restimulated 2 " " RMA-S-A2 (50 μl).

"

$ CTL assay

Expand & clone

Set up clones

Transfer to a well to 48 well plate

Feed

[redacted]

5

Sorting up: Self & Allo-restrict peptide specific CTL line

PBMC LG5 A2+ Self

LG6 A2+ Allo

peptides: WT-1 126. 187. 280
tyr control E7 86-95.

Methods, see previous page.

First round Stimulation with T2 loaded peptides.

Restimulate with peptide loaded-CIR A2

1L2 cl RMA-S-A2

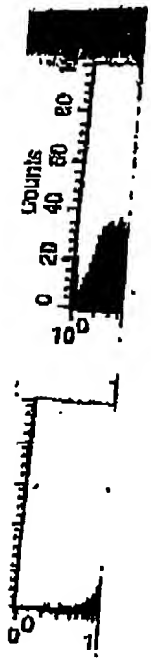
3L2 " " "

2L3 Restimulation CIR A2

7L3 CTL

2L3 L2 Cloning

4L7 CTL of clones



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Protocol for peptide specific allorestricted human CTL

1. Prepare stimulator cells: in case of T2 stimulators preincubate o/n 10^6 cells/ml/well in x24 well plates in RPMI medium plus 5% boiled (10 min) FCS with 100 uM peptide. If allo Dc are used as stimulators, allo DC from A2 +ve individuals can be separated as following:

Incubate A2+ PBMC at 37°C O/N, harvest nonadherent cells, resuspend in RPMI+9%FCS at $5X10^6$ /ml and layer onto metrizadime gradient carefully, spin at 1500 for 10 min and harvest the interface (30% low density DC, 20% recovered from PBMC counts). Wash X3 with medium, resuspend at 10 ^{6}/ml, add pepdides to a final conc of 50uM and incubate at 37°C for 4hr. (3000 rad) The stimulator can be added to responders directly w/O washing off peptides.

2. Separate blood by Ficoll centrifugation, wash twice, count, make sure that cells are HLA-A2 negative (by staining with HB54 and HB117 antibodies)

3. Plate $2x10^6$ effectors and $2x10^5$ stimulators per well in x24 well plates in culture medium (RPMI 1640, 10% FCS, 1% glutamine, 1% pen/strept $5x10^{-5}$ M 2-ME) with 500 nM peptide

4. On day 4 prepare stimulators as described in 1. if required. It may be not necessary to preincubate C1RA2 cells for more than 2 hrs

5. On day 5 harvest cells, count spin and seed $5x10^5$ effectors, $2x10^6$ irradiated (about 3000 rad) HLA-A2 negative autologous feeders, $2x10^5$ peptide loaded and irradiated (about 10000 rad) T2 or C1RA2 cells and 500 nM peptide in 2ml of the culture medium with 10u/ml IL-2, 2.5ng/ml IL7 (1/2000 of the stock of 5ug/ml) and with 10% of Q12054 culture sup (anti-CD4 antibodies) (complete medium)

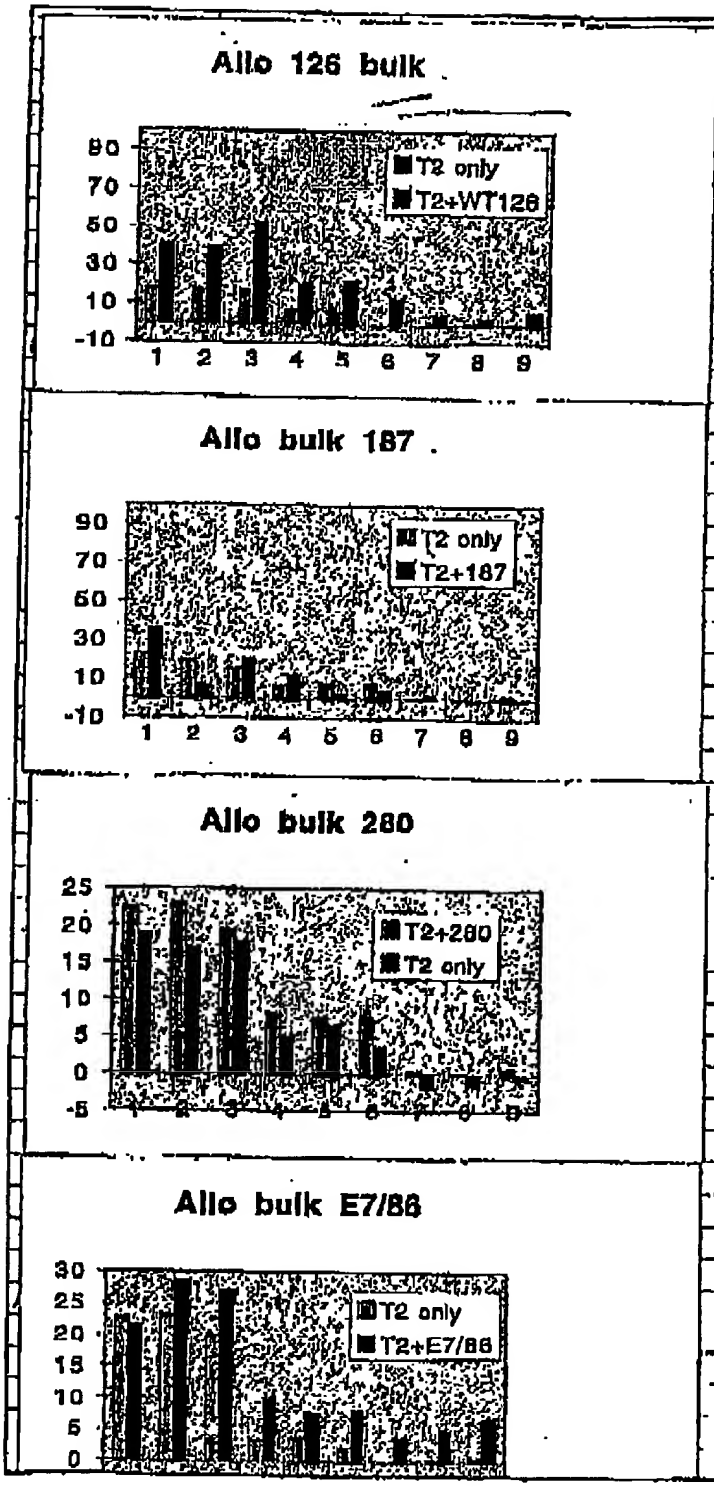
6. 14 days (and further every 2 weeks) later restimulate cultures as described in 5. Cell recovery is usually 200%

7. On day 5 analyse peptide specificity in a ^{51}Cr release assay using T2 or mouse cells transfected with A2 molecule as CTL targets and cold K562 cells as NK targets (50 per 1 labelled target). Use 0.7% TFA for max release instead of 0.5% SDS. Significant peptide specificity is detectable after 5-7 week in culture with E:T ratio 10:1

8. Peptide specific cultures can be cloned seeding 1, 10 and 100 cells/well in round bottom x96 well plates in complete culture medium with 10^4

(4)

Sheet1



	T2+126	
224	3199	303
284	3454	288
310	3290	51
300	3343	8
291	3168	
930	3332	
344	3262	
344	3261	
		4
	1534	41
	1508	40
	1870	52
	926	21
	970	22
	708	14
	456	5
	985	3
	492	6

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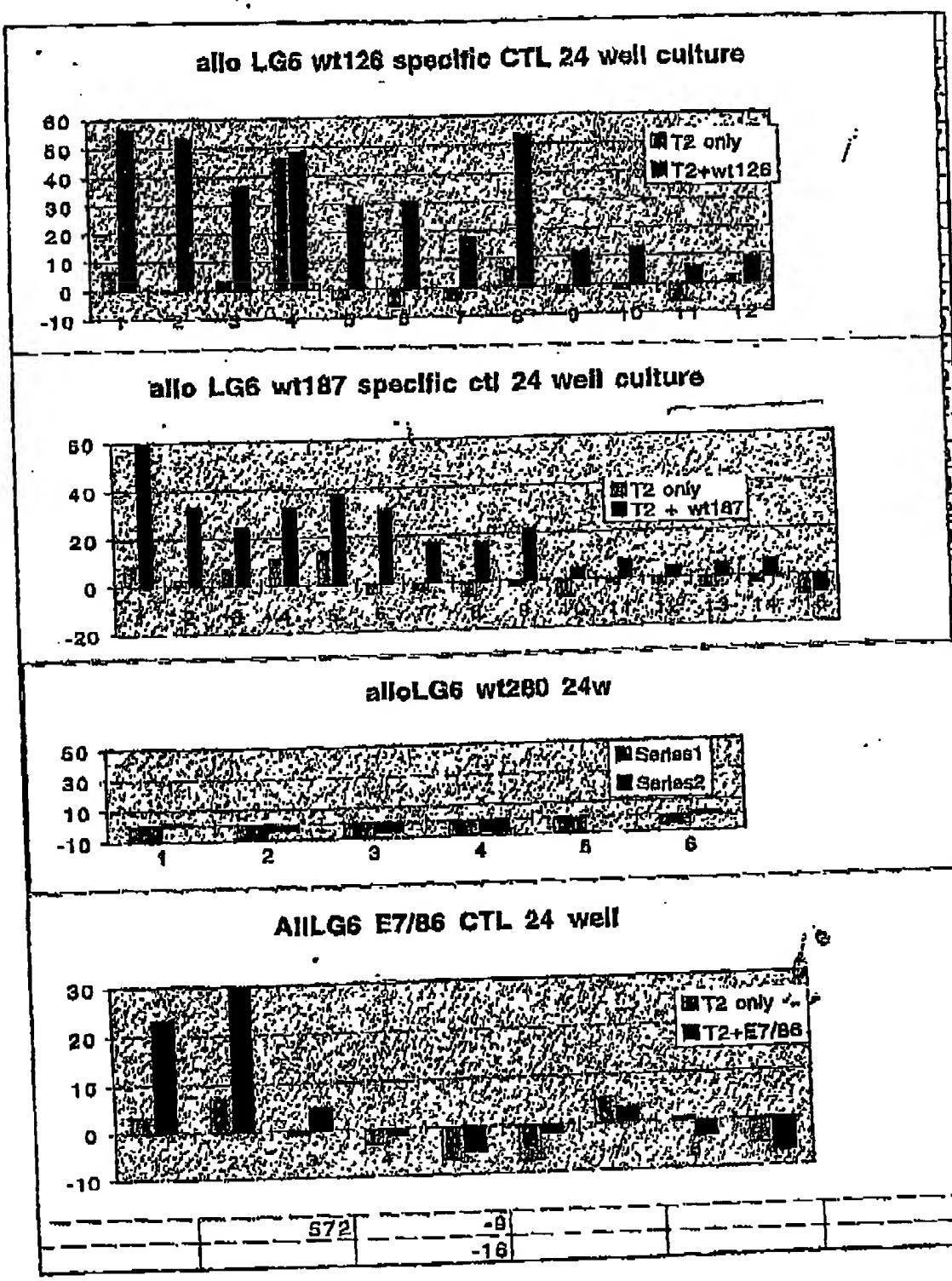
Sheet1


allo LG6 wt126 specific CTL 24 well culture
T2 only
T2+wt126
allo LG6 wt187 specific ctl 24 well culture
T2 only
T2 + wt187
alloLG6 wt280 24w
Series1
Series2
AllLG6 E7/86 CTL 24 well
T2 only
T2+E7/86

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31 Cr Release Assay

test clones Standard 4 hr

targets T2+E7
T2+126
K+62
K-62 Az

more tests

Sheet1

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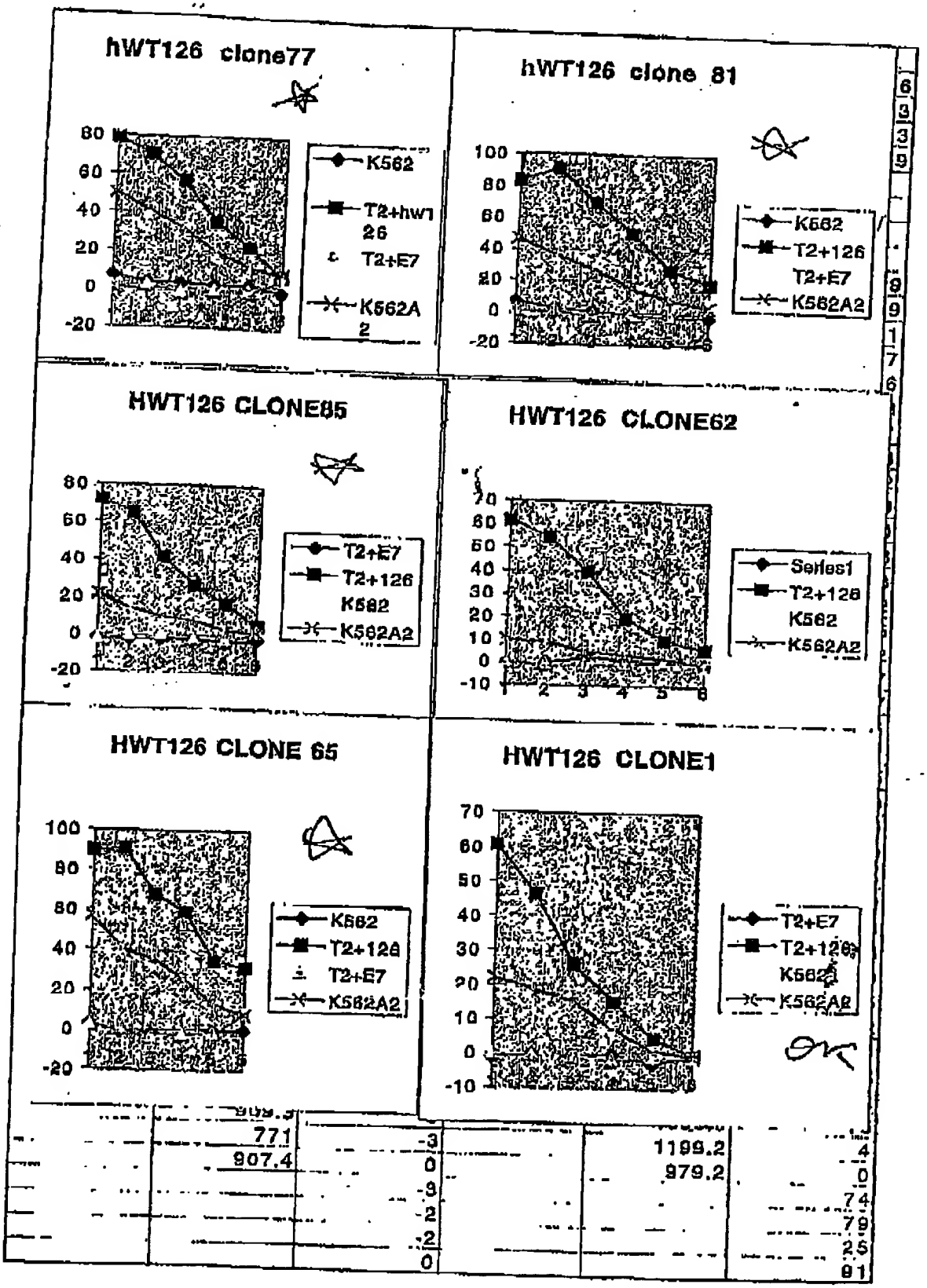
hWT126 clone77
K562
T2+hwt 26
T2+E7
K562A 2
hWT126 clone 81
K562
T2+126
T2+E7
K562A2
HWT126 CLONE85
T2+E7
T2+126
K562
K562A2
HWT126 CLONE62
Series1
T2+126
K562
K562A2
HWT126 CLONE 65
K562
T2+126
T2+E7
K562A2
HWT126 CLONE1
T2+E7
T2+126
K562
K562A2

7)

11

51 Cr assay

clone 77. 81. 32.

target: T2+E7 vs. control
T2+126
Br73.
MV 6041
697
CML Bone Marrow 4 (Foreman)
CD34+ from BM (by Stem)
E:T 25. 12. 6. 3. 1.5 0.75

Results:	line 77	line 32. *	81
T2+E3	-	+	+
T2+126	+	+	+
Br73	+	+	+
697	+	+	
MV4041	+	+	+
CML CD34+	+	+	-
CML BMφ	-	±	-

The clone 77 kills CD34+ from CML BM

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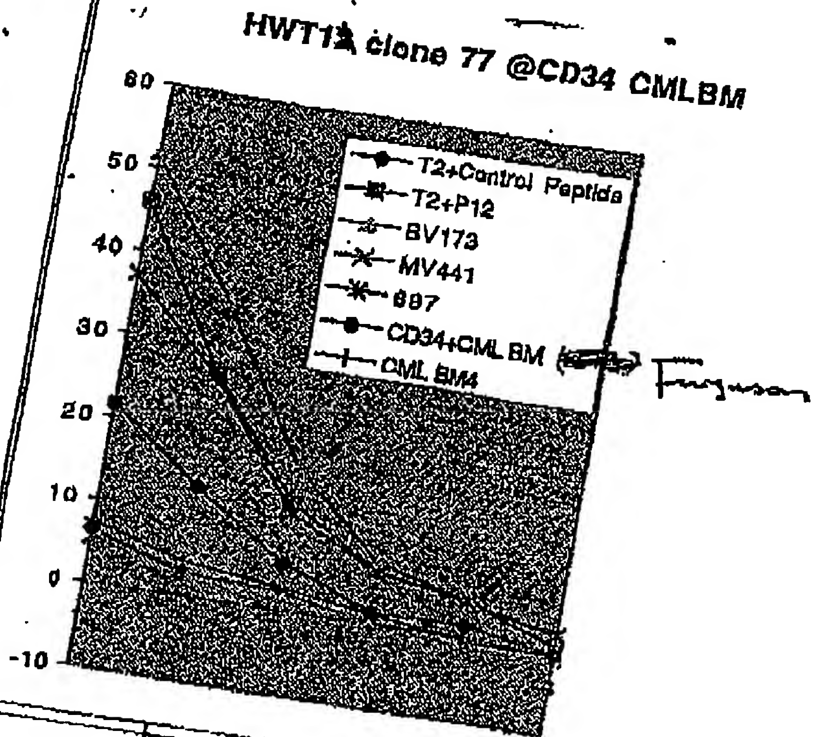

	41
	12
	10
	23
	27
	76
	69
	29
	80
	0
	11
	60
	68
	40
	31
	97
	97
	100
	105
	19
	117
	99
	100
-1	109
-4	25
-1	85
-1	102
28	61
57	86
19	99
50	87
-2	12
-2	61
-2	25
-4	101
-2	88
-1	124
67	81
13	85
6	26
3	116
0	26
-5	28
-5	46
-7	86
13	69
-3	1
-5	76

RNA RT PCR
697
Bv173 ++
Ferguson CD34+ +++
Thomas +
Gradley +
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